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EFFECT OF ORGANOPHOSPHORUS COMPOUNDS ON THE CONFORMATION OF ACETYLCHOLINESTERASE AND ACETYLCHOLINE RECEPTOR:

RECONSTITUTION OF GLOBULAR DIMER OF ACETYLCHOLINESTERASE AND INTERACTION OF ACETYLCHOLINESTERASE AND RECEPTOR WITH DIISOPROPYLFLUOROPHOSPHATE

Annual Report

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March 12, 1987

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5048

University of California
Laurel Heights, Suite 11
3333 California Street
San Francisco, California 94143-0962

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION University of California 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Laurel Heights, Suite 11 3333 California Street San Francisco, California 94143-0962	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-85-C-5048
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 62734A; PROJECT NO. 3M1-62734A875; TASK NO. AI; WORK UNIT ACCESSION NO. 373

11. TITLE (Include Security Classification)
(U) EFFECT OF ORGANOPHOSPHORUS COMPOUNDS ON THE CONFORMATION OF ACETYLCHOLINESTERASE AND ACETYLCHOLINE RECEPTOR

12. PERSONAL AUTHOR(S)
Yang, J.T., Wu, C.-S.C., Gan, L. and Reed, W.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 86/2/15 TO 87/2/14	87/03/12	42

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	01		Torpedo californica, acetylcholinesterase, acetylcholine receptor, diisopropyl fluorophosphate, activity, conformation, circular dichroism, sodium flux, RA 5
06	20		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The detergent-soluble globular dimer of acetylcholinesterase from Torpedo californica was reconstituted through dialysis into egg phosphatidylcholine vesicles. The size of the reconstituted particles depended on the ionic strength (I) of the buffer as well as the molar lipid/protein ratio (R). The solution of the protein-lipid complex was turbid at R = 5,000 and I = 0.13, and the particles became heterogeneous at R < 2,000. The enzyme was unstable at R = 1,000 and I = 0.05. Based on circular dichroism studies, the conformation of the enzyme reconstituted at R = 4,000 and I = 0.07 remained unaltered. The enzymatic activity and the Michaelis-Menten constant were also unchanged. The reconstituted enzyme seemed to be more stable against thermal denaturation than in detergent solution.

Acetylcholinesterase is irreversibly inhibited by diisopropyl fluorophosphate (DFP). The three isozymes, buffer-soluble globular dimer, asymmetric dodecamer and its derived

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian	22b. TELEPHONE (Include Area Code) (301) 663-7325
	22c. OFFICE SYMBOL SGRD-RMI-S

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

globular tetramer, had essentially the same bimolecular rate constant of 1.4-2.3 x 10^4 M^{-1} min^{-1}. The aging of DFP-inhibited globular tetramer had a half-life of 4.2-5 h. The number of DFP bound per globular monomer approached one at 60 μM DFP; the corresponding enzymatic activity dropped to near zero. Based on circular dichroic studies, there was no conformational change upon inhibition.

The acetylcholine receptor from Torpedo californica had virtually the same conformation in the absence and presence of DFP. Its binding affinity for α-bungarotoxin was also unaffected by the addition of DFP. Initial study of sodium flux through the receptor suggested that DFP might affect, in a slow, time-dependent manner, the accumulation of sodium ions.

Keywords: Sharks. (AW)

Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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Background

In the 1986 Annual Report (1) we described the preparation and purification of various isozymes of acetylcholinesterase (AChE) from Torpedo californica: the buffer-soluble globular dimer (G_2), asymmetric dodecamer containing a small amount of octamer (A_{12+8}) and its proteolytic product, the globular tetramer (G_4). The globular subunits of the three isozymes had essentially the same enzymatic activity and conformation (by circular dichroism [CD]). The stability of these enzymes against pH, thermal and urea denaturation was also similar to each other. We have now studied the enzymatic activity and conformation of detergent-soluble globular dimer reconstituted in lipid vesicles, which is described in Part I of this report. The enzymatic and conformational studies of native AChE are now completed. Thus we have begun to study the effects of organophosphorus (OP) compounds on the conformation of AChE. In Part II we report the effect of diisoproplyfluorophosphate (DFP). In Part III we report the preparation and purification of acetylcholine receptor (AChR) and some preliminary studies of the effect of DFP on the biological activity of AChR. The understanding of interactions of AChE and AChR with commercially available DFP will prepare us for the study of more potent OP compounds such as soman on these two proteins; this study will be initiated when soman is delivered to us.

Part I. Reconstitution of Detergent-soluble Globular Dimer of Acetylcholinesterase from Torpedo californica

Introduction

Acetylcholinesterase from Torpedo californica can be separated into three isozymes based on their solubilities (2-5). They are the low-salt-soluble globular dimer (LSS-G_2), detergent-soluble globular dimer (DS-G_2), and high-salt-soluble asymmetric dodecamer which contains a small amount of octamer (A_{12+8}). LLS-G_2 aggregated in the absence of detergent (5), but only DS-G_2 displayed the solubility characteristics of an integral membrane protein which is soluble in detergent solution. The size and structure of DS-G_2 from Torpedo californica resemble those of erythrocyte AChE. Both AChEs are dimers that are linked by a disulfide bond; each subunit has a small hydrophobic domain that interacts with lipid vesicles (6-9). The hydrophobic domain of erythrocyte AChE can be removed by papain without affecting the size, shape and enzymatic activity of the enzyme (10), but the papain-treated enzyme can no longer incorporate into liposomes (11). This hydrophobic domain is a covalently linked glycolipid at the COOH-terminus of the enzyme (12). Similarly, the hydrophobic domain of Torpedo DS-G_2 can be removed by phosphatidylinositol-specific phospholipase C, Pronase K and pronase E, but not papain (6). Little is known about the chemical nature of this removed portion of the Torpedo AChE.

The reconstitution of erythrocyte AChE into liposomes has been studied extensively. Only a small portion of the protein molecule is inserted into the liposome and there is no specificity for the head group of the phospholipid (8). Aggregated AChE does not bind to preformed liposomes (9-11); most of the incorporated enzyme is found on the exterior surface of the liposome and can thus be degraded by papain (9-11). Cardiolipin was reported to be tightly bound to the erythrocyte AChE (13).

In contrast, little has been reported on the reconstitution of AChE from electric organs. The asymmetric AChE binds to sphingomyelin but not phosphatidylcholine (14-15), probably through the collagen-like tail of the enzyme. DS-G_2 can be incorporated into phosphatidylcholine liposomes (6, 16).

In most reconstitution studies of AChE, the size of liposomes is not a cause for concern. But large liposomes introduce undesirable artifacts due to light scattering, which makes it difficult to interpret optical measurements such as CD (17). To date, there are no practical and effective methods for correcting such artifacts.

In this report we studied factors that affect the size of the reconstituted AChE and found that it depended on the molar lipid/protein ratio and the ionic strength of the buffer used in reconstitution. Under suitable conditions, a clear solution

of the reconstituted DS-G_2 can be obtained. The reconstituted AChE in buffered solution changed neither the conformation nor the Michaelis-Menten constant of DS-G_2 in detergent solution, but it seemed to be enzymatically more stable against thermal denaturation than the enzyme in detergent solution.

Materials and Methods

Materials. Liquid-nitrogen-frozen electroplax of Torpedo californica was obtained from Marinus, Long Beach, CA. The non-ionic detergent dodecyl octaoxyethylene ether ($C_{12}E_8$) was from Nikkol of Japan. Cholic acid (Sigma Chemical Company, St. Louis, MO) was recrystallized from ethanol. L-α-Phosphatidylcholine from egg yolk (egg PC, type IX-E), E. coli β-galactosidase, bovine catalase, 1-amino-2-naphthol-4-sulfonic acid were purchased from Sigma. Hydrogen peroxide (30%) and Sephacryl S-400 were purchased from Fisher Scientific, Santa Clara, CA and Sigma, respectively.

Preparation of DS-G_2. After LSS-G_2 was removed with low-salt buffer (18), the electroplax tissue was homogenized again in a buffer of 0.2 M NaCl/0.02 M Tris/0.02 M $MgCl_2$ (pH 7.5) in 1:2 buffer volume to tissue weight (ml/g). An equal volume of 2% sodium cholate was then added. The solution was stirred on ice for 1 h, and centrifuged for 30 min at 45,000 rpm in a Beckman 45 Ti rotor at 4°C. The supernatant was loaded on an acridine-Sepharose affinity column (18) preequilibrated with 1% sodium cholate/0.1 M NaCl/0.01 M Tris/0.01 M $MgCl_2$ (pH 7.5) and eluted with the same buffer containing 10 mM decamethonium bromide. The concentration and enzymatic activity of the purified DS-G_2 were determined by the methods described previously (18).

Preparation of phosphatidylcholine vesicles. Lipid vesicles were prepared by the method of Huang and Charlton (19) with minor modifications. Two hundred mg of egg PC was dissolved in 10 ml of chloroform-methanol (2:1) in a round-bottom flask and dried on a rotary evaporator to generate a thin, homogeneous film of the lipid. After overnight evacuation with a vacuum pump to remove traces of organic solvents, the lipid was resuspended in 5 ml water and sonicated in a bath sonicator (Laboratories Supplies, Model G112SP1T) at 50-60 Hz for 1 h in ice bath. The solution was then centrifuged in a Beckman SW 50.1 rotor at 35,000 rpm and 4°C for 1 h. The pellet was discarded; portions of the supernatant were used to determine the lipid concentration.

Reconstitution of DS-G_2. Purified DS-G_2 in 0.1 M NaCl/0.01 M Tris/0.01 M $MgCl_2$ (pH 7.5)/1% sodium cholate was dialyzed for 2 h at 4°C against the same buffer but without sodium cholate to lower the molar ratio of cholate to protein to about 2,000 to 1 (the volume of the buffer used was based on dialysis equilibrium after 2 h). Reconstitution was achieved by adding an appropriate amount of preformed egg PC vesicles to the dialyzed DS-G_2 solution, which gave the desired molar lipid/protein ratio. After incubation for 1 h at 4°C, the reconstituted DS-G_2 solution was

dialyzed against a buffer of 0.01 M Tris/0.01 M $MgCl_2$ (pH 7.5)/ NaCl (added to the desired ionic strength). The dialysis buffer was changed once over a period of 20-24 h. Sucrose density gradient centrifugation of the reconstituted DS-G_2 in the same buffer used in dialysis was then performed as described previously (18).

Determination of inorganic phosphate. The concentration of the lipid was determined by its inorganic phosphate content according to a modified method of Bartlett (20). Briefly, 20-400 µl samples were placed in Pyrex ignition tubes; to each tube was added 0.3 ml of 11.3 N H_2SO_4, 0.3 ml of 30% H_2O_2, and a boiling chip. The tubes were heated in a test-tube heater at 240°C for 30 min. If the solution was still unclear, additional 0.3 ml of 30% H_2O_2 was added; the tubes were heated for another 15 min. After cooling to room temperature, 5 ml of distilled water was used to wash down the tube wall. Next, 0.2 ml of 3% ammonium molybdate and 0.2 ml of a reducer solution (7.5% $NaHSO_3$ /0.2% Na_2SO_3/0.125% 1-amino-2-naphthol-4-sulfonic acid) was added to each tube. The tubes were then kept in a boiling-water bath for 30 min or more until a blue color was well developed. The concentration of the inorganic phosphate was determined by its absorbance at 830 nm in a Zeiss spectrophotometer (dibasic sodium phosphate was used as the standard). The ratio of inorganic phosphate to egg PC was 1:27.8 (w/w).

Circular dichroism. All CD spectra were measured on a JASCO J-500A spectropolarimeter as described previously (18).

Amino acid composition. Amino acid analysis was done at the Department of Biochemistry and Biophysics, University of California at Davis.

Results

Characterization of DS-G_2. The size, chemical composition and enzymatic properties of DS-G_2 showed many similarities to those of LSS-G_2 reported previously (18). Both enzymes had a sedimentation coefficient, s, of 5.7S in 1% Triton as determined by sucrose density gradient centrifugation, but DS-G_2 in 1% sodium cholate had a slightly higher s value (6.5-6.8S), probably due to difference in size of the detergent micelles. The amino acid compositions of DS-G_2 and LSS-G_2 were virtually indistinguishable from each other (Table I); they also agreed with that of LSS-G_2 (18). The Michaelis-Menten constant, K_M, for both enzymes was 40 µM (with acetylthiocholine as the substrate), but the maximum velocity, V_{max}, varied from preparation to preparation for either enzyme with no detectable trend. Reconstituted DS-G_2 in egg PC also had the same K_M as DS-G_2 in detergent solution.

Size of reconstituted DS-G_2. The size of reconstituted DS-G_2 in egg PC depended not only on the molar lipid/protein ratio (R) but also on the ionic strength (I) of the buffer for dialysis to

remove the detergent. In general, the peaks in the sedimentation pattern shifted from left to right with increasing R values (Fig. 1). That is, reconstituted DS-G_2 sedimented more slowly at higher R values because of low density of the lipid, even though the protein-lipid complex became bigger. At a constant I of 0.05, the reconstituted DS-G_2 sedimented as a sharp, single peak (Fig. 1, column A). Further, the peaks for enzymatic activity overlapped those for the lipid content, indicating that the protein was incorporated 100% to the liposome with no free lipid or free protein in the solution.

A similar dependency of the size of reconstituted DS-G_2 on R was found at I = 0.13 (Fig. 1, column B). The peak at R = 2,000 (Fig. 1, B-2) appeared to be broader than that at R = 4,000 (Fig. 1, B-3), and the peak at R =1,000 was highly heterogeneous (Fig. 1, B-1), probably because of aggregation of the protein.

The effect of ionic strength of the dialysis buffer on the particle size of reconstituted DS-G_2 was further studied at R = 5,000 (Fig. 2). As the ionic strength increased from 0.05 to 0.07 (Fig. 2A and B), the peak of the complex shifted from left to right. At I = 0.13 (Fig. 2C), the protein-lipid complex floated at the top of the centrifuge tube. A similar preparation subjected to gel filtration on a Sephacryl S-400 column was eluted at the void volume, indicating the protein-lipid complex was enormously large. However, the size of the reconstituted DS-G_2 could vary from preparation to preparation even under identical conditions. In one experiment with R = 5,000 and I = 0.13, the protein peak sedimented at 4S and the solution appeared to be clear. Thus we routinely checked the reconstituted samples by sucrose density gradient centrifugation.

Conformation of reconstituted DS-G_2. A major problem for reconstitution is turbidity of the buffered solution of reconstituted DS-G_2. From the results in Figs. 1 and 2, we found that particles with an s value between 4S and 11S generally formed clear solutions suitable for CD measurements. We chose R = 4,000 based on the results in Fig. 1 and I = 0.07, which was a compromise because the enzyme became unstable if the ionic strength was too low. The protein-lipid complex in this buffered solution showed a CD spectrum close to that of DS-G_2 in the nonionic detergent $C_{12}E_8$ solution (Fig. 3). The results also agreed with the CD spectrum of LSS-G_2 in $C_{12}E_7$ solution as reported previously (18). Thus, reconstitution of DS-G_2 into egg PC vesicles did not appear to alter its conformation (the distortion of the CD spectrum due to light scattering seemed to be insignificant in this case). Analysis of the CD spectra in Fig. 3 by the method of Chang et al. (21) and Yang et al. (22) gave 40% α-helix, 40% β-sheet and 4% β-turn for the reconstituted AChE and 37% α-helix, 42% β-sheet and 0% β-turn for the same protein in detergent solution. These differences were within experimental errors of the current method of analysis.

Temperature dependency of enzyme activity. The relative

enzyme activity of reconstituted DS-G_2 in PC vesicles first increased with increasing temperature from 100 at 4°C to 325 at 35°C and then declined steeply to zero at 50°C (Fig. 4). On the other hand, the relative activity of DS-G_2 in sodium cholate solution rose only to 180 from 4°C to 25°C and then dropped to zero at 40°C. Thus, reconstituted enzyme in phospholipid solution appeared to be more stable than the same protein in detergent solution against thermal denaturation.

In another experiment both reconstituted DS-G_2 in phospholipid solution and DS-G_2 in sodium cholate solution were incubated at 30°C up to 6 h. The enzyme activity of the reconstituted protein remained stable regardless of the concentration used (0.2 mg/ml or 0.01 mg/ml) (Fig. 5). The same was true for DS-G_2 at 0.2 mg/ml in detergent solution. However, reducing the concentration of the enzyme to 0.01 mg/ml destabilized the protein, which began to denature after 20-min incubation; and its activity dropped to 20% after 1 h. These results again suggested that the reconstituted DS-G_2 was thermally more stable than DS-G_2 in detergent solution.

Discussion

Reconstitution of a membrane protein typically begins with a mixed micellar solution of the purified protein, detergent, and lipid. Various methods in the literature differ in the timing of the addition of lipid and the method of removing the detergent. Lipid can be added as mixed lipid-detergent micelles or as preformed vesicles during solubilization, during purification on the chromatographic column, or after the protein is purified. The most common method of removing the detergent is by dialysis, gel filtration, and dilution, the choice of which depends on individual proteins. Ideally, the end product is a suspension of unilamellar vesicles of uniform size with a uniform lipid to protein ratio.

The size and shape of the reconstituted protein depend on R, and are often critical for the assay of functions of integral membrane proteins. For ion-conducting proteins such as AChR and sodium-channel protein, large unilamellar vesicles with measurable inner volumes are preferred. Reconstituted AChR at an R value of 300 shows predominantly bilayer sheets with no trapped inner volume (23). Unilamellar vesicles suitable for ion-conducting studies are formed when R is 1,000 or larger. On the other hand, Klausner et al. (24) were able to reconstitute functional asialoglycoprotein receptor at R = 130 and 520. These reconstituted products sediment at different rates in KBr density gradient centrifugation. Apparently, only a small area of this asialoglycoprotein interacts with the lipid bilayers because the trapped dye inside the vesicle does not leak out when the protein is bound to the vesicle. The DS-G_2 of AChE also possesses a small hydrophobic domain that interacts with the lipid (6); therefore, large liposomes may not be necessary for the protein

to function.

We were able to reconstitute DS-G_2 into egg PC at an R value of 1,000 and an ionic strength of 0.05, but the protein was unstable at this ionic strength. Increasing the ionic strength to 0.13 resulted in aggregation of the protein, which did not bind with the lipid. Thus the choice of an ionic strength between 0.05 and 0.1 represented a compromise. At low R values, e.g., 1,000, there were not enough liposomes for the protein to contact. In addition, the decreased surface charge of the protein at a high salt concentration might have promoted self-aggregation through the hydrophobic domain of the protein and, in turn, prevented the protein from interacting with the liposome. This is supported by the observations that aggregated AChE does not bind to preformed liposomes (9-11). A high lipid-protein ratio favored the formation of large reconstituted particles (Fig. 1), but it did not affect the binding of DS-G_2 to the lipid. Our choice of R = 4,000 appeared to be satisfactory for optical measurements. Little is known about the effect of ionic strength on the size of reconstituted proteins. It is likely that increasing the counter ion concentration reduces the electrostatic repulsion between the head groups of PC and allows association of lipid monomers into larger liposomes.

The CD spectra showed that no significant conformational change was associated with the reconstitution of DS-G_2 to egg PC vesicles. The enzymatic properties were also unaffected. But the liposomes of the reconstituted DS-G_2 appeared to enhance the stability of the enzyme against thermal denaturation. Unlike other membrane-transversing proteins, the portion of the AChE molecule inserted into the lipid bilayer is small and probably distant from the active site. It is thus difficult to imagine how the lipid can influence the stability at the active site. However, sodium cholate is known to inactivate erythrocyte AChE (7,11). Therefore, observed difference in stability is probably due to the destructive effect of the detergent in dilute AChE solutions rather than to the protective effect of the lipid to the reconstituted enzyme.

The size, composition and enzymatic properties of detergent- and low-salt-soluble AChE are extremely similar; yet one is extractable only in the presence of a detergent, and the other can be obtained easily by low-salt extraction. This difference may be due to the difference in the hydrophobic domain of the two enzymes so that DS-G_2 interacts more strongly with the membrane than LSS-G_2. On the basis of pronase digestion studies, Witzemann and Boustead (25) have suggested that the difference between low-salt- and detergent-extractable AChE is confined to the outer structure of these molecules. This question can be answered when the primary structures of these proteins become available.

Part II. Interaction of Acetylcholinesterase

Introduction

Organophosphorus (OP) compounds phosphorylate serine at the active site of acetylcholinesterase (AChE) and irreversibly inactivate the enzyme. The enzymatic activity can be restored by dephosphorylation of the enzyme with quaternary pyridine aldoximes. However, aging occurs when a prolonged inactivation of the enzyme spontaneously and progressively transforms the enzyme to a form that is resistant to reactivation (27-29). The rate of aging depends on the alkyl or alkoxy group on the OP compound (30); it is more rapid for a secondary, tertiary, or cyclic alkyl group and slower for a primary alkyl group. The aging of AChE that has been inactivated by DFP closely correlates with the removal of one of the isopropyl groups (31). The rate of aging of soman-inactivated AChE is essentially identical with the rate of dealkylation, and the reaction is catalyzed by an acidic group in the enzyme with a pK_a of 6.4 (32). On the other hand, a base-catalyzed dealkylation has also been proposed (33). In either case a negative charge introduced after dealkylation at the active site is thought to be an electrostatic barrier to the attack by oximes (34). Coulombic repulsion between the negatively charged phosphoro group and the anionic subsite of the enzyme also expands the distance between the anionic subsite and the active center of the aged AChE (35). Fluorescent study suggests that the phosphoro group is more deeply buried inside the active site for the aged enzyme-inhibitor complex than for the non-aged complex (36).

We report here the kinetics of inhibition of AChE by DFP. We determined the bimolecular reaction constant, k_i, for various isozymes of AChE from Torpedo californica and the rate of aging and the binding isotherms of the enzyme with DFP. Circular dichroism study showed no difference in conformation between native and aged AChE.

Materials and Methods

Materials. Liquid nitrogen-frozen electroplax of Torpedo californica was obtained from Marinus, Long Beach, CA. Three forms of AChE--G_2, A_{12+8} and its proteolytic product, G_4--were prepared and purified on acridine-Sepharose affinity columns as described previously (18). DFP (Sigma Chemical Company, St. Louis, MD) and [1,3-^{3}H]-DFP (DuPont/NEN Products, Boston, MA) were used without further purification. 2-Pyridinealdoxime methiodide, 1-pyrenesulfonamidoethyltrimethylammonium iodide and DE81 filter discs were purchased from Aldrich Chemical Company, Milwaukee, WI, Molecular Probes, Eugene, OR and Whatman, Hillsboro, OR, respectively. Edrophonium chloride and propidium iodide were from Sigma.

Kinetics. The bimolecular reaction constant, k_i, between AChE and DFP was calculated from equation (1):

$$k_i = \frac{2.30\log(v_1/v_2)}{(t_2 - t_1)[I]} \qquad (1)$$

where v_1 and v_2 are enzyme activities at times t_1 and t_2 and [I] is the concentration of the inhibitor. Enzyme activity was determined by the method of Ellman et al. (26). The rate of aging of DFP-inactivated AChE was determined by the procedure of Beauregard et al. (37).

Binding isotherm. About 1 μM AChE was incubated with [1,3-^{3}H]-DFP at each of various concentrations (10 to 60 μM) in 100 μl buffer (10 mM sodium phosphate and 20 mM NaCl at pH 7.5) for 1 h at room temperature. Twenty μl each was then spotted on a DE81 filter disc, washed twice with 300 ml buffer, and counted on a Beckman LS-100 scintillation counter. An unwashed spotted disc gave the total counts for bound and unbound DFP and washed discs spotted with different concentrations of [1,3-^{3}H]-DFP but without AChE served as the blanks. Bound DFP per AChE (mol/mol) was calculated from equation (2):

$$\frac{\text{Bound DFP}}{\text{AChE}} = \frac{[\text{DFP}]}{[\text{AChE}]} \times \frac{(\text{washed count} - \text{blank})}{\text{total count}} \qquad (2)$$

Protein concentrations were determined by the method of Lowry et al. (38); the molecular weight of a subunit was taken as 70,000. The activity of AChE for each incubated solution was determined concurrently.

Circular dichroism. CD spectra were measured on a JASCO J-500 spectropolarimeter at 25°C under nitrogen flush. The data were expressed as mean residue ellipticity, [θ], in deg cm^2 dmol^{-1}.

Results and Discussion

Inhibition. The mode of inhibition of AChE by DFP is exponential (Eq. 1); a log activity vs. time plot for each DFP concentration yields a straight line. The bimolecular reaction constant, k_i, is calculated from the slopes of the linear plots divided by the concentration of DFP. The three isozymes of AChE seemed to show some differences in their k_i value (Table II). These differences, however, may not be significant because the k_i value varied from one preparation to another. For instance, one preparation of G_2 gave a k_i value as low as 0.8×10^4 M^{-1} min^{-1} and another preparation of A_{12+8}, as high as 3.6×10^4 M^{-1} min^{-1}. Our k_i value for G_4 was close to $1.7\text{-}1.8 \times 10^4$ M^{-1} min^{-1} for G_4, obtained by Gordon et al. (39) with purified DFP, but slightly higher than 0.95×10^4 M^{-1} min^{-1} as determined by Forsberg and Puu (40), who introduce a reversible enzyme-inhibi-

tor complex preceding an irreversible step (see Eq. 5 below). Because of the similarities in enzymatic and conformational properties as well as the bimolecular reaction constant, k_i, for G_2, G_4 and A_{12+8} of AChE, we chose G_4 for all other experiments in this report.

The half-life of aging of DFP-inactivated AChE was 4.2-5 h and the rate of aging 0.14-0.17 h^{-1}. Our results of the half-life were slightly shorter than those found by Beauregard et al. (37). Since the half-life should be a constant, a faster aging rate might indicate the presence of a contaminating inhibitor that reacts with AChE, and ages faster than DFP.

Binding isotherm. The binding of [1,3-^{3}H]-DFP to G_4 of AChE was a slow process as compared with that of other OP compounds such as soman. The binding increased with time and approached a plateau after 40 min. Thus, for routine experiments the mixture of AChE and DFP was incubated for 1 h to attain a maximum binding but without the complications due to aging. Prolonged incubation would cause a decrease in the count of radioactivity because of the dealkylation of one of the two radioactive isopropyl groups of DFP. This opposite effect would lead to an apparently lower degree of binding, even though the number of DFP molecules bound to AChE remained unchanged. The binding isotherm showed a limit of one mole DFP bound per mole of AChE and the corresponding enzymatic activity dropped to zero at 60 μM [1,3-^{3}H]-DFP (Fig. 6). Because the reaction is irreversible, the binding data were not treated as multiple equilibria with identical and independent sites (the Scatchard plot). In another experiment, AChE was incubated with 4 μM [1,3^{3}H]-DFP for 20 min and then with cold DFP for 4 h. The radioactivity of the AChE-DFP complex decreased with increasing amount of cold DFP and about one half of the hot DFP was displaced by 40 μM or more cold DFP. Whether the binding is partially reversible remains to be investigated.

The mechanism of irreversible inhibition of AChE by OP compounds has been proposed to proceed in two steps with the formation of a Michaelis-Menten-type complex preceding the irreversible step (27,40,41):

$$\text{AChE-OH} + \text{IX} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{AChE-OH}\cdot\text{IX} \xrightarrow{k_2} \text{AChE-OI} + \text{HX} \qquad (3)$$

where AChE-OH represents the enzyme and IX the inhibitor with a leaving group X. The bimolecular reaction constant, k_i, is related to the other constants by

$$k_i = k_2(k_1/k_{-1}) \qquad (4)$$

or

$$k_i = k_2/K_d \qquad (5)$$

where K_d is the dissociation constant for the reversible enzyme-inhibitor complex. For the inhibition of AChE by DFP, Forsberg and Puu found a k_2 of 0.17 s^{-1} and K_d of 1.1 mM, which gives a k_i of 0.93×10^4 M^{-1} min^{-1} (40), as mentioned earlier. With the binding of soman to AChE, these authors reported a K_d of 61 μM and a k_i of 5.6×10^7 M^{-1} min^{-1}, suggesting that this OP compound has a higher affinity for the enzyme and is a more potent inhibitor than DFP.

Conformation. DFP-inhibited G_4 of AChE had a slightly smaller magnitude of mean residue ellipticities than native G_4 in both the far- and near-UV regions (Fig. 7). This small difference in the CD spectra was within experimental errors. Further, the CD spectrum of DFP-inhibited G_4 was time independent even 24 h after the addition of DFP. Thus, the binding of DFP to the enzyme did not seem to change the conformation of AChE. The same was true for the addition of edrophonium chloride (data not shown), which is known to bind to the anionic subsite at the active center. On the other hand, fluorescence studies by Amitai et al. (36) have shown that the aged conjugate produced by reacting AChE with 1-pyrenbutyl phosphorodichloridate has a larger quantum yield and a smaller quenching constant then the non-aged conjugate of AChE with 1-pyrenbutylethyl phosphorochloridate. They concluded that the aged conjugate has the phosphoro group more deeply buried inside the active center of the enzyme than its non-aged counterpart. Thus, CD may be less sensitive than fluorescence in detecting small local conformational changes in this case. Our CD results in the 300-nm region (Fig. 2, inset), which monitors the tryptophan chromophores, remained unchanged upon the binding of DFP, even though the Try residue(s) is believed to be near the active center of AChE (18, 42,43).

In addition to the anionic site at the active center, AChE possesses a peripheral anionic site which binds many large ligands such a propidium, tubocurarine and gallamine. This peripheral site has been shown to affect the enzymatic activity (44) and the conformation at the active center (45) of AChE. Therefore, we believe that a ligand that binds to the peripheral site and also induces extrinsic CD in the near-UV and visible regions may be used as a probe for the difference, if any, between the aged and non-aged AChE. Our preliminary study with propidium iodide failed to show any induced CD in the near-UV region and 1-pyrenesulfonamidoethyltrimethylammonium chloride induced a small CD band around 350 nm. However, both compounds have high absorption coefficients in the near-UV and visible regions, thus making CD measurements difficult. Work is in progress to search for other suitable ligands to test our idea.

Part III. Interaction of Acetylcholine Receptor with Diisopropyl Fluorophosphate

Introduction

The nicotinic AChR has been characterized in great detail with respect to its structural and biochemical features (46). The abolition of normal nervous activity attended by OP compounds such as DFP, soman, sarin and tabun has been ascribed to their inhibition of AChE. Because of the putative similarity between the active site of AChE and the binding site for acetylcholine on the AChR, an interaction between OP compounds and AChR is probable. Three functions of AChR have been studied with purified AChR inserted in well-defined vesicles (47) and these studies provide instances, beside structural analysis, to study the effects of OP compounds on the AChR. These functions are (a) agonist binding, (b) ion-channel activation and (c) desensitization which blocks ion permeability. But little is known about the effects of OP compounds on these functions. In this brief report, we show the conformational change, or the lack of it, for AChR in the presence of DFP and also preliminary studies of the binding of α-bungarotoxin and ion flux by the addition of DFP.

Materials and Methods

Materials. Liquid nitrogen-frozen electroplax of Torpedo californica was purchased from Marinus, Long Beach, CA. Buffers used were:

Buffer A--10 mM phosphate, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and 0.02% sodium azide, pH 7.5

Buffer B--10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 100 mM NaCl, 0.1 mM EDTA and 0.02% sodium azide, pH 7.4

Buffer C--buffer B plus 1% final sodium cholate, pH 7.4

Buffer D--buffer C plus 1 mg/ml dioleoylphosphatidylcholine (DOPC).

Purification of AChR. AChR was prepared from Torpedo californica and purified by affinity chromatography on an acetylcholine-derivativized sulfhydryl-terminal agarose gel, essentially according to the method of McNamee and coworkers (47-49). Briefly, about 200 g of frozen electroplax tissue was ground and homogenized. Iodoacetamide (2 mM) and phenylmethylsulfonyl fluoride (PMSF) (0.1 mM) were added as protease inhibitors. The homogenate was centrifuged at 5,000 g for 10 min. The supernatant was filtered through cheesecloth and then centrifuged at 105,000 g for 60 min to pellet the crude membrane fraction. The pellets were resuspended in buffer B and then frozen in liquid

nitrogen and stored at -70°C until further treatment. This crude membrane fraction was enriched in AChR as revealed by electron microscopy and by the binding of ^{125}I-α-bungarotoxin. It was solubilized in buffer C with stirring at 4°C. The suspension was then centrifuged at 105,000 g for 45 min to remove the insoluble material and the supernatant was applied to the affinity column prepared from Affi-gel 401 (Bio-Rad) and bromoacetylcholine, which was synthesized from choline bromide and bromoacetyl bromide (50). The affinity column was washed extensively with buffer D. Purified AChR was eluted with buffer D containing carbamylcholine at a 10 mM final concentration. The peak fractions, based on absorbance at 280 nm, were pooled. Three kinds of lipid vesicles were used. The pooled fractions were (i) dialyzed directly to give AChR in DOPC vesicles, (ii) added to an asolectin-cholate solution and dialyzed, or (iii) added to a cholate suspension of DOPC, dioleoylphosphatidic acid (DOPA) and cholesterol at a molar ratio of 75:15:10 and dialyzed. All dialysis was carried out against buffer B at 4°C. Protein concentrations were determined by the method of Lowry et al. (38). Amino acid analysis and the Lowry assay agreed within 10%.

SDS-PAGE. Slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 5% stacking gel and a 10% resolving gel of polyacrylamide. The gels were removed after electrophoresis and stained with Coomassie Blue to determine the integrity of the AChR preparations.

Circular dichroism. CD spectra of AChR with or without DFP were measured on a JASCO J-500A spectropolarimeter. The data were expressed as mean residue ellipticities [θ] and analyzed (22).

Toxin binding. Triton X100-solubilized AChR was incubated with an excess of ^{125}I-α-bungarotoxin in the absence or presence of 5 μM carbamylcholine and with or without DFP. The procedure was essentially that described by Fong and McNamee (47), except that the DE-81 filters were thrice washed by batch washes.

Ion flux assay. The influx of ^{22}Na ions into AChR was measured in the range of seconds as described by Yee et al. (49). AChR (50 μl) and ^{22}Na ions in buffer B with or without 10 μl carbamylcholine (about 1 mM) were incubated for 30 sec on ice. A 50 μl aliquot was filtered on a Dowex 50W-8X column and washed with the buffer. The elute was then counted.

Results and Discussion

Circular dichroism. The far-UV CD spectra, indicative of the secondary structure of biopolymers, were examined for AChR in the vesicles of DOPC (Fig. 8) and asolectin (data not shown) at a molar lipid/protein ratio of about 400. The spectra in the absence and presence of DFP (100 to 500 μM) were virtually iden-

tical; the observed small difference in the 210-220 nm region (< 10%) may not be significant. CD analysis of the data (51) indicated that AChR had 40-50% α-helix, about 20% β-sheets and 10-20% β-turns. Our results were similar to those reported for unperturbed AChR (e.g., Ref. 51). The near-UV CD spectra, which monitor the aromatic chromophores of AChR with and without DFP, likewise reflected no difference (within 1%) in the mean residue ellipticities.

Since the addition of DFP did not elicit changes in CD spectra in the far- and near-UV regions, any conformational change due to the interaction of DFP with AChR could be of insufficient magnitude to be revealed by CD. Alternatively, the lack of any conformational change in AChR in the presence of DFP might be due to the particular vesicles used, which precluded interaction between the protein and DFP, although this possibility seems to be unlikely.

One consideration for CD measurements was the choice of the molar lipid-protein ratios. At high ratios, e.g., 10,000 or higher, the lipid vesicles were too turbid for CD measurements. Yet these high ratios were needed for other studies such as sodium flux. Even at a low molar ratio of about 400, the problem of light scattering still existed and it could distort the CD spectra and thereby any quantitative analysis.

Toxin binding. The binding of ^{125}I-α-bungarotoxin to AChR in the vesicles made up of DOPC, phosphatidic acid and cholesterol at a molar ratio of 75:15:10 was not sufficiently affected by the presence of 500 μM DFP. In this experiment the ^{125}I was substantially decayed and the lack of difference in the binding could have been due to the breakdown of the toxin. In another experiment with fresh ^{125}I-α-bungarotoxin and AChR in asolectin vesicles, still no significant difference in the binding could be detected in the absence and presence of DFP. In other preliminary studies, the time course of toxin binding from 30 sec to 150 sec did not appear to be particularly different.

The lack of any effect of DFP on the binding of α-bungarotoxin to AChR may be that the sites of interaction for the toxin and DFP are different or that perhaps rates of the binding of antagonist or possibly agonist (e.g., carbamylcholine) are affected but not the final stoichiometries. This needs to be addressed with carbamylcholine and its effect on ion flux and desensitization in future work. Our preliminary work seems to suggest that ion accumulation may be reduced by the addition of DFP. Work is planned to use other well defined lipid vesicles and also higher protein concentrations to focus on this aspect of the function of AChR.

Sodium ion flux. Initial study of $^{22}Na^{+}$ ion flux through AChR in asolectin vesicles suggested that the addition of about 300 μM

DFP may affect, in a slow, time-dependent manner, the accumulation of sodium ions with a progression of about 30% by 30-sec coincubation to 60% by 20-min preincubation.

SDS-PAGE. The examination of the AChR by electrophoresis on SDS-PAGE showed primary stained bands generally having molecular weights of 39,000, 48,000, 51,000 and 64,000. These values were consistent with the known molecular weights of the two α-, one each β-, γ- and δ- subunits of AChR. Our preparations also showed little proteolytic fragments.

Table I. Amino Acid Composition of Detergent- and Buffer-soluble Globular Dimer of Acetylcholinesterase

Residue	Detergent-soluble G_2	Buffer-soluble G_2
Asx	12.78	11.7
Thr	5.3	4.9
Ser	8.2	8.0
Glx	9.3	9.8
Pro	5.6	6.1
Gly	9.3	9.0
Ala	5.3	6.1
Val	6.6	7.0
Met	1.4	1.2
Ile	3.9	3.9
Leu	9.5	9.5
Tyr	4.2	4.2
Phe	7.0	6.3
His	2.5	2.8
Lys	4.6	5.1
Arg	4.8	4.7

The numbers are expressed in mol % and calculated from analyzed amino acid residues.

Table II. Bimolecular Reaction Constant (k_i) for Various Forms of Acetylcholinesterase

Isozymes	AChE μg/ml	DFP μM	$k_i \times 10^{-4}$ M^{-1} min^{-1}	Average k_i M^{-1} min^{-1}
G_2	13	1.96	1.51	
		2.94	1.29	
		3.92	1.51	
		5.88	1.23	
		7.84	1.21	1.35×10^4
G_4	14	0.98	1.66	
		1.96	1.67	
		2.94	1.47	
		3.92	1.56	1.59×10^4
A_{12+8}	23	0.98	2.36	
		1.96	2.29	
		2.94	2.12	
		3.92	2.40	2.29×10^4

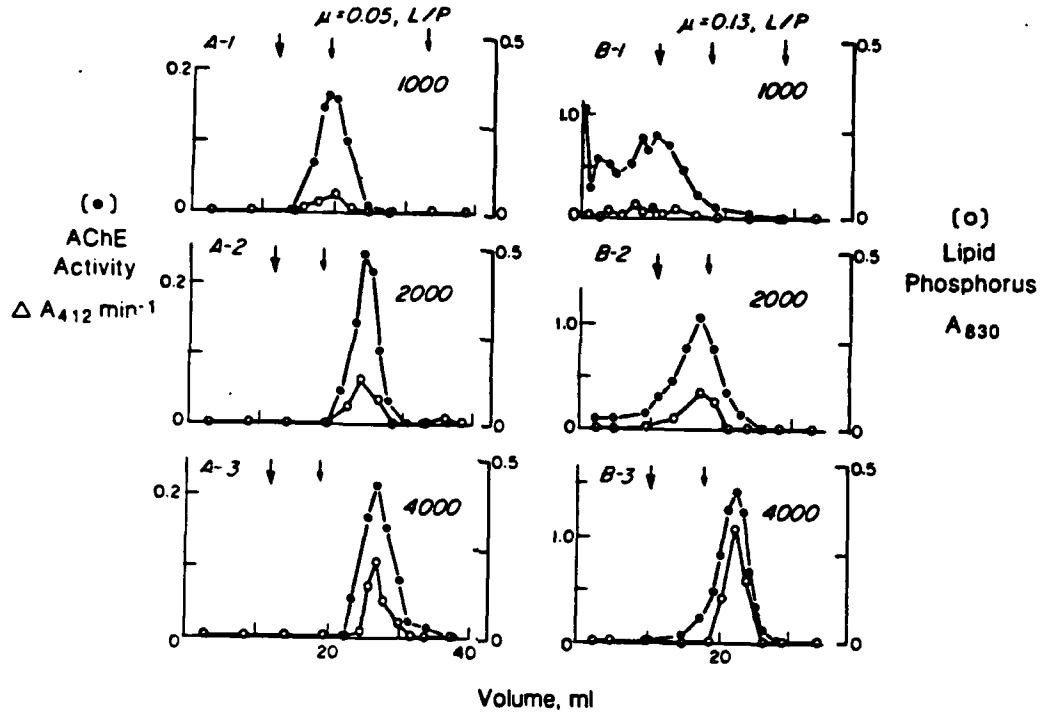

Figure 1. Effect of molar lipid/protein ratio on the sedimentation pattern of reconstituted detergent-soluble globular dimer of acetylcholinesterase. Molar lipid/protein ratios: A-1 and B-1, 1,000; A-2 and B-2, 2,000; A-3 and B-3, 4,000. Ionic strength: A's, 0.05; B's, 0.13. Symbols: ●, enzyme activity; 0, inorganic phosphate. Sucrose density gradient centrifugation was carried out at 4°C in 5-25% sucrose gradient. Buffer: 0.01 M Tris/0.01 M $MgCl_2$ (pH 7.5) and NaCl added to the desired ionic strength. Direction of sedimentation: from right to left. Markers: light arrow, catalase (11.4S); heavy arrow, β-galactosidase (16S).

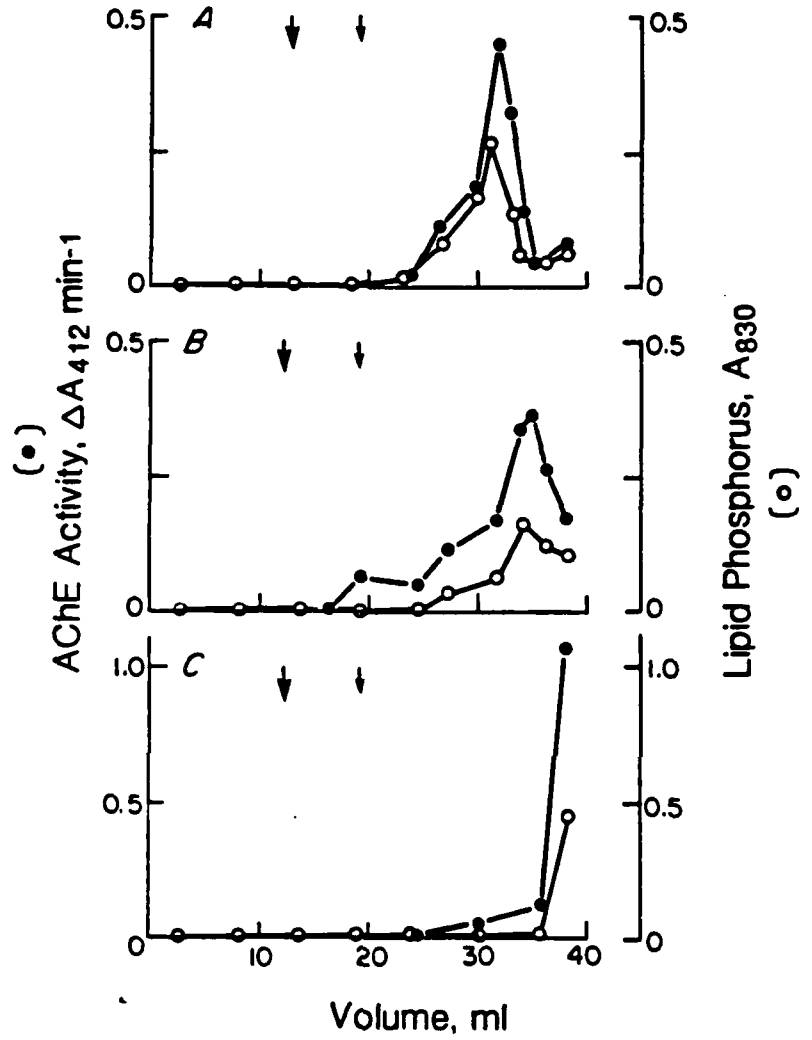

Figure 2. Effect of ionic strength of the dialysis buffer on the sedimentation pattern of reconstituted detergent-soluble acetylcholinesterase. Ionic strength: A, 0.05; B, 0.07; C, 0.13. Molar lipid/protein ratio: 5,000. Conditions and symbols for sucrose density gradient centrifugation were the same as those in Fig. 1.

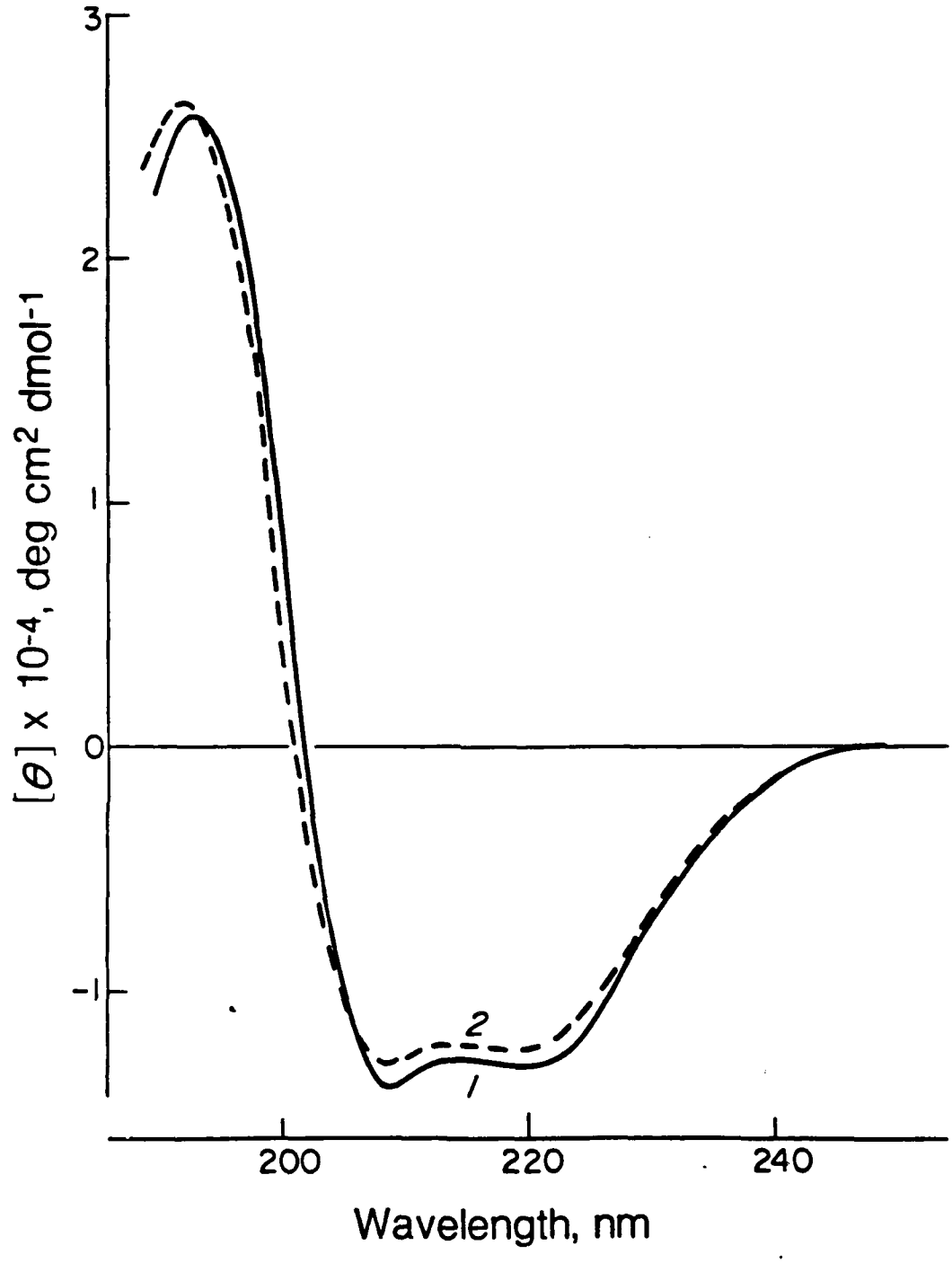

Figure 3. Circular dichroism of detergent-soluble acetylcholinesterase in lipid vesicle and detergent solutions at 4°C. Curves: 1, in phosphatidylcholine vesicle (molar lipid/protein ratio: 4,000); 2, in dodecyl octaoxyethylene ether. Buffer: 0.01 M sodium pyrophosphate/0.002 M $MgCl_2$ (pH 7.5).

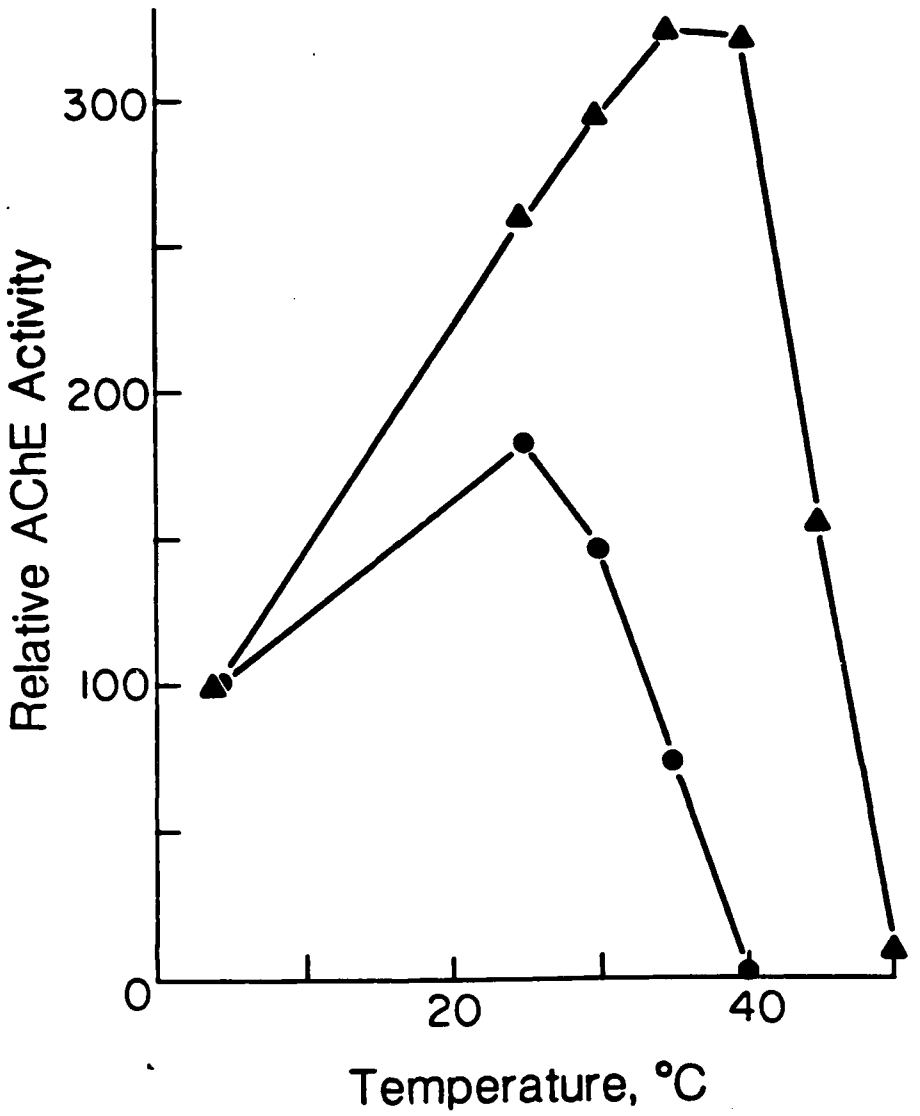

Figure 4. Effect of temperature on the relative activity of detergent-soluble acetylcholinesterase in lipid and detergent solutions. Symbols: ▲, in phosphatidylcholine solution (molar lipid/protein ratio: 5,000); ●, in 1% sodium cholate solution. The enzymatic activity at 4°C was taken as 100. Stock solutions of the enzyme (0.2 mg/ml) in lipid vesicle and in 1% sodium cholate were diluted ten times with 0.1 M sodium phosphate/ 0.005 M $MgCl_2$ (pH 7.5). Aliquots of 10 µl of each solution were assayed for their enzyme activity by the method of Ellman et al. (26) at indicated temperatures.

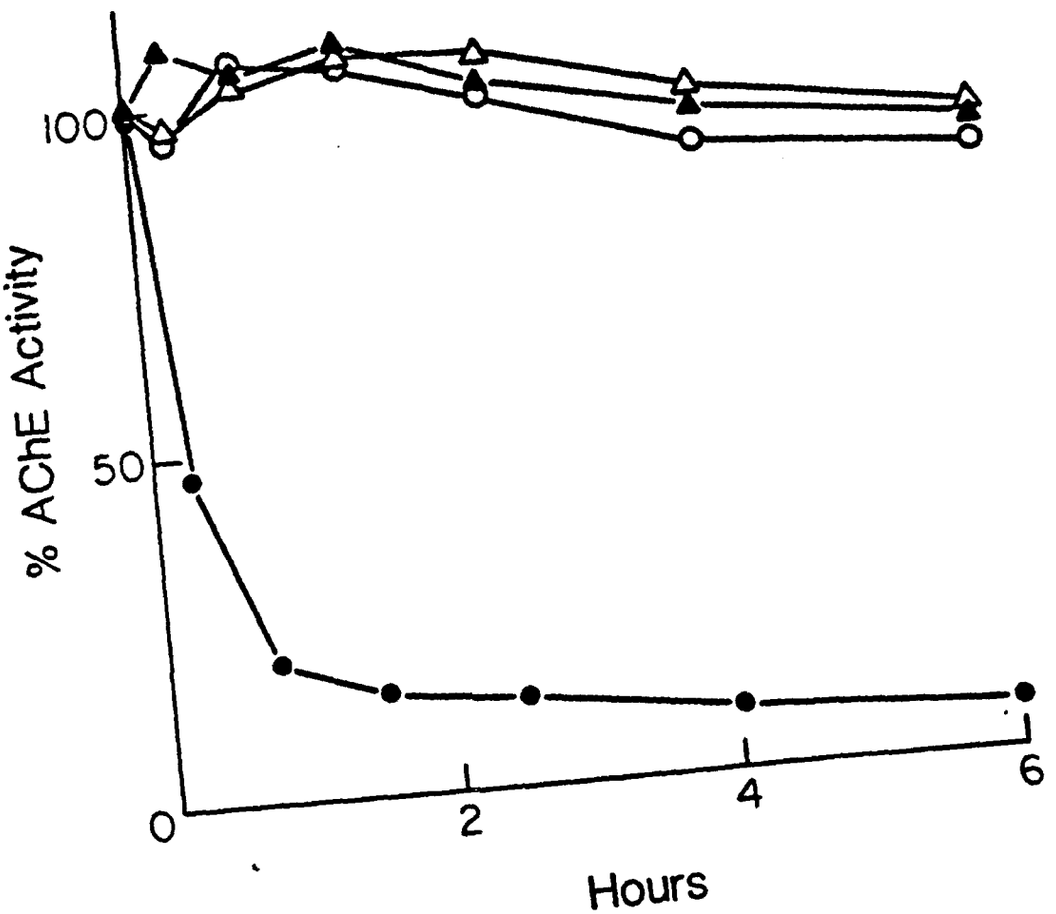

Figure 5. Thermal stability of detergent-soluble acetylcholinesterase in lipid and detergent solutions at 30°C. The enzyme in phosphatidylcholine vesicle (molar lipid/protein ratio: 5,000) or 1% sodium cholate was incubated at 30°C. Aliquots of the samples were taken at intervals for enzyme assay by the Ellman method at 23°C. Concentrations of the enzyme: Δ, 0.20 mg/ml in lipid solution; ▲, 0.01 mg/ml in lipid solution; 0, 0.23 mg/ml in detergent solution; ●, 0.01 mg/ml in detergent solution.

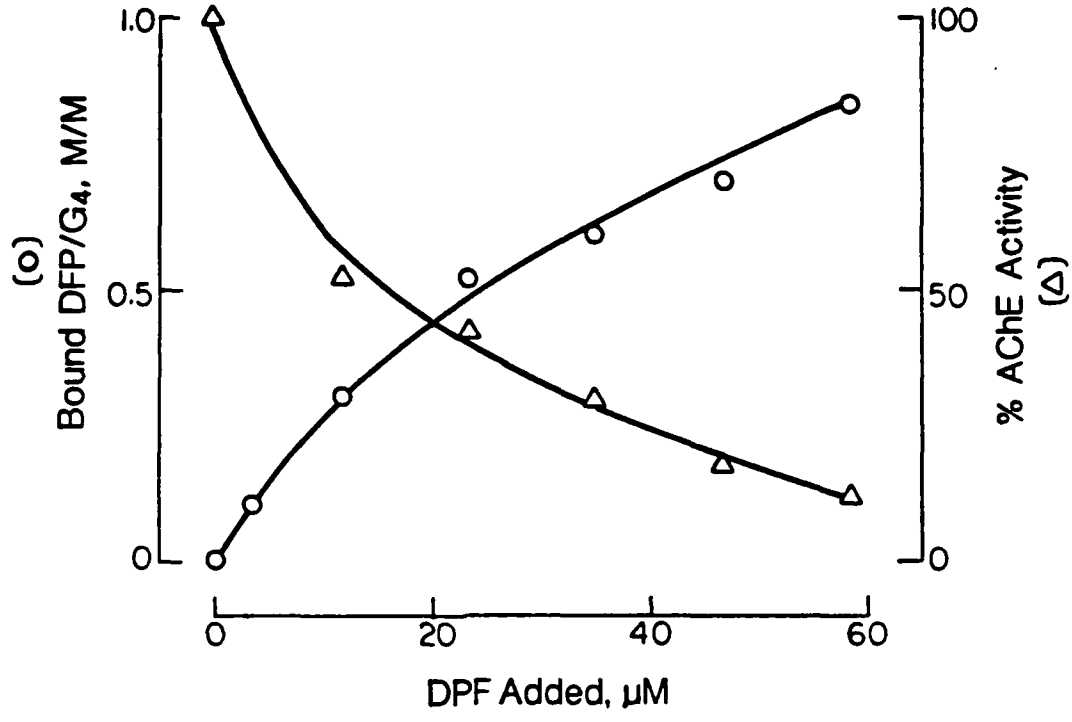

Figure 6. Binding isotherm of diisopropyl fluorophosphate to acetylcholinesterase at room temperature. Seven hundred forty nM of globular tetramer was reacted with 10 to 60 μM [1,2-^{3}H]-DFP in 100 μl buffer (10 mM sodium phosphate plus 20 mM NaCl, pH 7.5) for 1 h. Twenty μl aliquots were spotted on DE81 filter discs, washed twice in 300 ml buffer, and counted on a Beckman LS-100 scintillation counter. Enzyme activity was determined by the method of Ellman et al. (26). DFP bound to AChE was calculated according to Eq. (2).

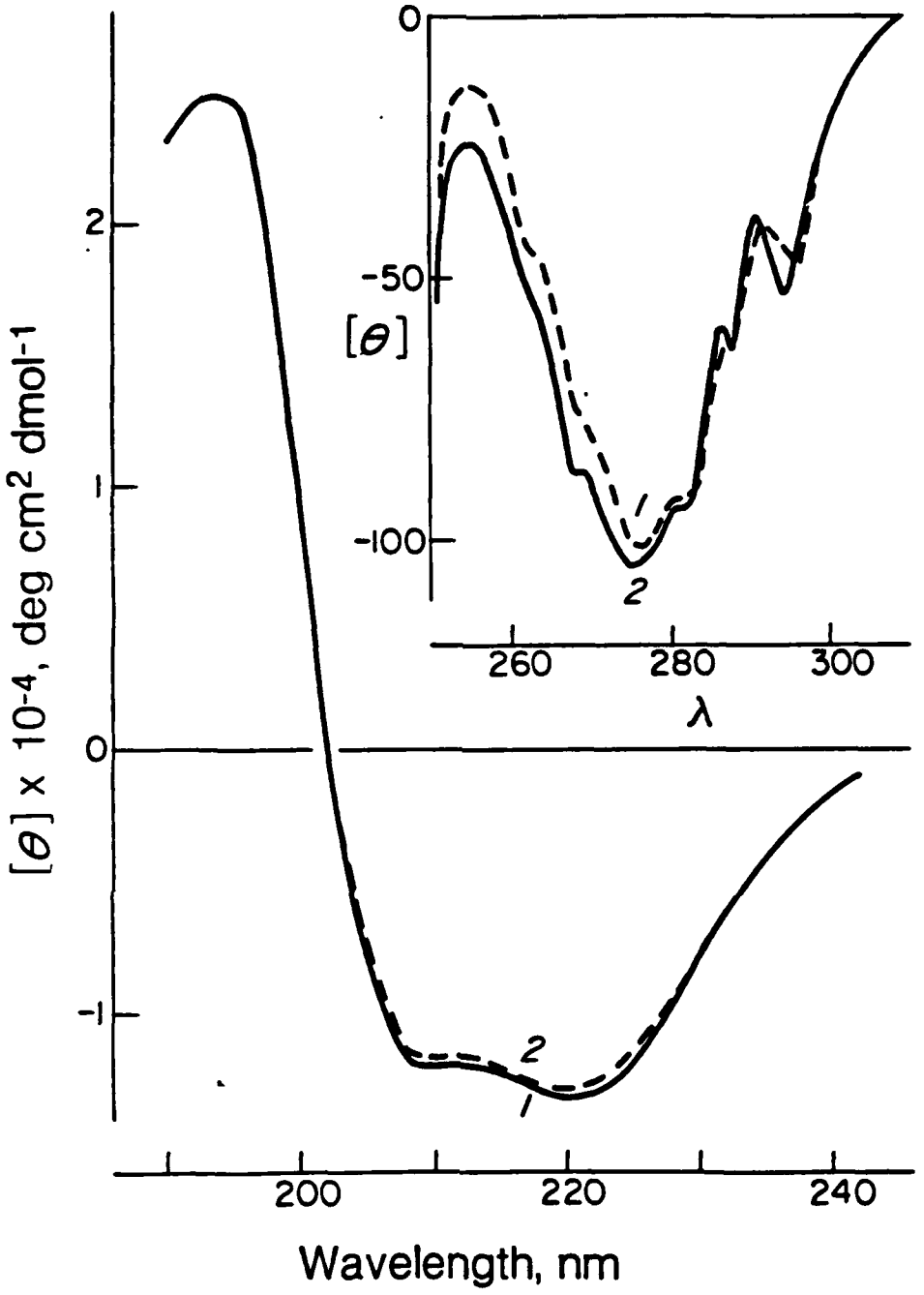

Figure 7. Circular dichroic spectra of native and aged acetylcholinesterase at 25°C. Curves: 1, native AChE; 2, aged AChE. AChE was aged by reacting with 100 μM DFP for 4 h at room temperature. The enzymatic activity dropped from 3.4 mmol min^{-1} mg^{-1} to zero after incubation. Protein concentration: 0.36 mg/ml. Buffer: 10 mM Tris, 0.1 M NaCl and 10 mM $MgCl_2$ at pH 7.5.

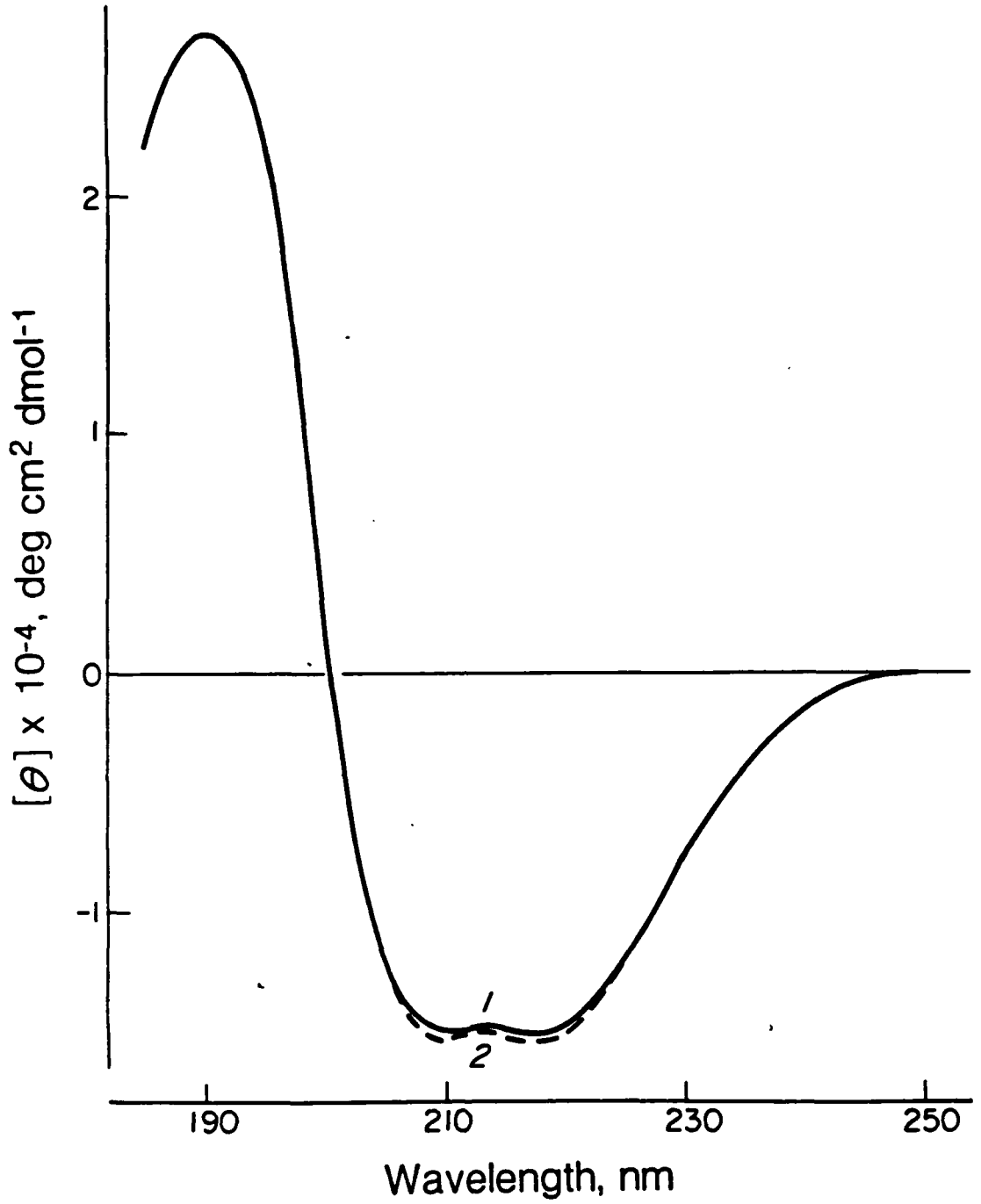

Figure 8. Circular dichroic spectra of acetylcholine receptor in the presence of diisopropyl fluorophosphate at 25°C. Curves: 1, zero DFP; 2, 250 μM. The receptor was incorporated into DOPC vesicles at a molar lipid-protein ratio of less than 1,000. The solvent consisted of DOPC vesicles in a buffer containing 10 mM MOPS, 100 mM NaCl, 0.1 mM EDTA and 0.02% sodium azide (pH 7.4). DFP was added and diluted in the same buffer. Protein concentration: 0.30 mg/ml.

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Glossary

A_{12+8}	asymmetric dodecamer of AChE containing a small amount of octamer
AChE	acetylcholinesterase
AChR	acetylcholine receptor
CD	circular dichroism
$C_{12}E_7$	dodecylheptaoxyethylene ether
$C_{12}E_8$	dodecyloctaoxyethylene ether
DFP	diisopropyl fluorophosphate
DOPA	dioleoylphosphatidic acid
DOPC	dioleoylphosphatidylcholine
DS	detergent-soluble
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
G_2	globular dimer
G_4	globular tetramer
I	ionic strength
K_M	Michaelis-Menten constant
LSS	low-salt-soluble
MOPS	3-(N-morpholino)propanesulfonic acid
OP	organophosphorus
PC	phosphatidylcholine
PMSF	phenylmethylsulfonyl fluoride
R	molar lipid-protein ratio
S	Svedberg unit (1S = 10^{-13} sec)

SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
UV	ultraviolet
V_{max}	maximum velocity

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